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cDNA cloning of a germ cell specific lamin B_3 from mouse spermatocytes and analysis of its function by ectopic expression in somatic cells

K.Furukawa and Y.Hotta

Department of Biology, School of Science, Nagoya University, Nagoya 464-01, Japan

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The nuclear lamina is a fundamental component involved in the assembly of the nuclear envelope and higher order chromosomal structures in eukaryotes. In mammals, it is composed of four major lamin proteins, termed lamins A, B_1, B_2 and C. Here we first report cDNA cloning of a new 53 kDa lamin protein from mouse spermatocytes, termed lamin B_3, the expression of which appears restricted to spermatogenic cells. Its gene structure indicates that lamin B_3 is generated by differential splicing and alternative polyadenylation from lamin B_2. When lamin B_3 is introduced into somatic cells in culture, their nuclear morphology is transformed from spherical to hook-shaped. On the basis of the results obtained, we suggest that the germ cell specific lamin B_3 is involved in the reorganization of nuclear and chromosomal structures during meiotic division.

Key words: lamin B_3/meiosis/nuclear morphology/spermatocyte/synaptonemal complexes

Introduction

Lamins are major karyoskeletal proteins which form a filamentous meshwork on the nucleoplasmic surface of the inner nuclear membrane. They physically separate the nucleoplasm from the cytoplasm and have important functions in maintaining the integrity of the nuclear envelope and the higher order chromosomal structure during mitosis (reviewed in Gerace and Burke, 1988; Nigg, 1989; McKeon, 1991).

Two distinct lamin protein types, A- and B-type, have been identified in higher vertebrates. B-type lamins, which maintain an association with nuclear membrane vehicles during metaphase in mitosis, are involved in anchoring the lamina to the nuclear membrane, while A-type lamins, which assemble at the chromosome surfaces, contribute to the organization of the interphase chromatin (Gerace and Blobel, 1980; Stick *et al.*, 1988; Krohne *et al.*, 1989; Burke, 1990; Glass and Gerace, 1990; Kitten and Nigg, 1991). In mammals, the nuclear lamina of somatic cells is composed of four major lamin proteins, termed lamins A, B_1, B_2 and C. Lamins A and C have almost identical amino acid sequences except for their C-terminal domains, and probably arise by differential splicing of RNA from one gene (Fisher *et al.*, 1986; McKeon *et al.*, 1986; Riedel and Werner, 1989). In contrast, lamins B_1 and B_2 are encoded by two distinct genes (Höger *et al.*, 1988, 1990).

The expression of lamin proteins is regulated in a tissue- and development-dependent fashion (reviewed in Krohne and Benavente, 1986). Undifferentiated embryonal carcinoma cell lines and early embryos both only express B-type lamins. A-type lamins first appear when differentiation is induced or development of organs is commenced (Lebel *et al.*, 1987; Stewart and Burke, 1987; Röber *et al.*, 1989). In *Xenopus*, germ cell specific lamins L_{III} and L_{IV} were found in diplotene oocytes and spermatids, respectively (Stick, 1988; Benavente and Krohne, 1985). In the chicken, however, only lamins A, B_1 and B_2, and no germ cell specific type, are detected in diplotene oocytes (Lehner *et al.*, 1987). In pachytene gametocytes undergoing meiosis, no lamin proteins have ever been detected in either of these two animals (Stick and Schwarz, 1982, 1983; Lehner *et al.*, 1987). Up to now, however, it has remained unclear whether any nuclear lamin proteins are expressed in mammalian meiotic cells (Stick and Schwarz, 1982; Schatten *et al.*, 1985; Maul *et al.*, 1986; Longo *et al.*, 1987; Moss *et al.*, 1987; Kaufmann, 1989; Sudhakar and Rao, 1990; Höger *et al.*, 1990).

Morphological examinations using the electron microscope have indicated that mammalian pachytene cells have a different nuclear architecture from somatic cells. The nuclei of spermatocytes show a closely aggregated distribution of nuclear pore complexes in specific portions of the nuclear envelope during the long prophase in meiosis (Fawcett and Chemes, 1979). The pairing of homologous chromosomes requires assembly of meiosis-specific fibrillar structures such as the synaptonemal complexes (Counce and Meyer, 1973; Heyting *et al.*, 1985). Not all of the components of these synaptonemal complexes have yet been identified but some are present in the nuclear matrix fraction (Heyting *et al.*, 1987; Sudhakar and Rao, 1990; Smith and Benavente, 1992), implying that a germ cell specific nuclear lamina might be present in pachytene nuclei.

To address this question, we reinvestigated mouse spermatocytes using Western and Northern blot analyses, and here report cloning of a mammalian germ cell specific B-type lamin, termed lamin B_3. The cDNA and amino acid sequences of lamin B_3 indicate that it is generated by differential splicing and alternative polyadenylation from lamin B_2. Structural and functional differences from lamin B_2 suggest, however, that the germ cell specific lamin B_3 may play important roles in organizing meiotic cell nuclear architecture.

Results

Western blot analysis of infant and adult testes and spermatocytes

Western blot analysis of total cell extracts from infant and adult testes, purified pachytene spermatocytes and nuclear matrix fractions from adult testes and spermatocytes using a specific monoclonal antibody raised against rainbow trout lamins (anti-fish lamin-specific antibody), showed that lamins A (70 kDa), B (B_1, 67 kDa and B_2, 67 kDa) and C

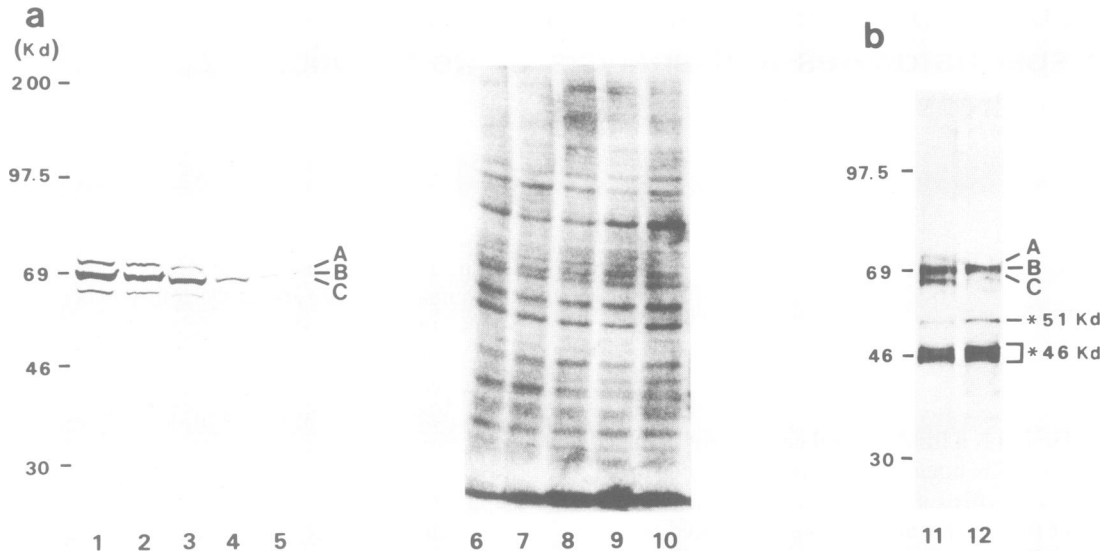

Fig. 1. Detection of tissue and cell specific lamin proteins by Western blot analysis. Total cell extracts (shown in **a**) and/or nuclear matrix fractions (shown in **b**) were prepared from testes of 7 (lanes 1 and 6), 14 (lanes 2 and 7), 21 (lanes 3 and 8) and 70 day old mice (lanes 4, 9 and 11) and purified spermatocytes (lanes 5, 10 and 12). The total cell extract proteins (50 μg) and nuclear matrix proteins (5 μg) were electrophoresed on SDS−polyacrylamide gradient gels (4−20%) and analyzed by Western blotting with anti-fish lamin-specific monoclonal antibody (lanes 1−5, 11 and 12). Proteins were visualized by staining with Coomassie blue (lanes 6−10). Lamin A and C proteins are marked by A and C, respectively, and lamin B_1 and B_2 proteins are marked by B. Asterisks indicate the newly identified lamin-related 51 kDa protein and the ~46 kDa proteins.

(65 kDa) were all expressed by both infant and adult testes. However, the expression of these proteins decreased in testes during postnatal development (Figure 1; panel a, lanes 1−4 and panel b, lane 11), suggesting that the amounts of these lamins are dependent on maturation of spermatogenic cells during testicular development. Indeed, we found that spermatocytes only produced B-type lamins, at very low levels (Figure 1; panel a, lane 5 and panel b, lane 12). Moreover on SDS−PAGE, the nuclear matrix fraction from spermatocytes was found to contain additional proteins immunologically related to lamins: a single band of ~51 kDa and multiple bands at ~46 kDa (Figure 1b). These proteins, which were recognized by an antibody against rat lamin B but not by those specific to lamins A and C, were not detected in the liver nuclear matrix fraction (data not shown).

Northern blot analysis of spermatocytes, testes and COP5 cells

We further investigated poly(A)$^+$ mRNAs of cultured mouse COP5 cells as representative somatic cells, as well as adult testes and purified spermatocytes, by Northern blot analysis with the cDNAs of mouse lamins B_1 or B_2 (Höger *et al.*, 1988, 1990). Both lamin B_1 (2.9 kb) and lamin B_2 (3.5 kb) mRNAs could be detected in testes and spermatocytes but at one-fifth and one-tenth respectively of the levels found in COP5 cells (Figure 2a and b). Surprisingly, one additional ~1.8 kb mRNA was found in spermatocytes using lamin B_2 cDNA, but this band was never detected in COP5 mRNAs (Figure 2b). Therefore the 1.8 kb mRNA was concluded to be a germ cell counterpart of lamin B_2.

In contrast, lamin A and C mRNAs were detected in COP5 cells and whole testes, but not in spermatocytes by Northern blot analysis with a 37 base oligonucleotide of lamin C (Figure 2c). Lamins A and C were regulated at the transcriptional level, with only low expression in adult testes and no expression in spermatocytes. These results are in complete agreement with the Western blot analysis

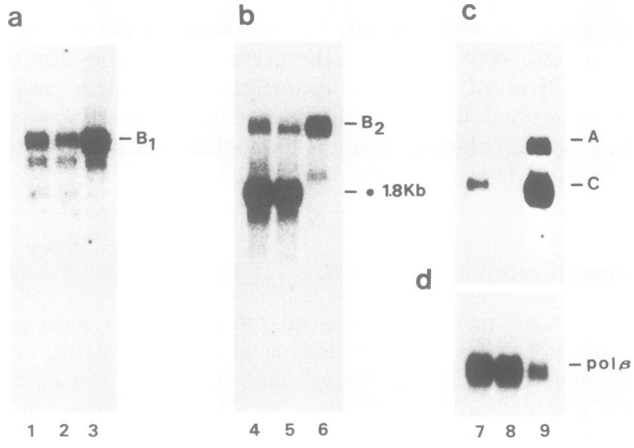

Fig. 2. The detection of lamin B_2-related mRNA in spermatocytes by Northern blot analysis. Poly(A)$^+$ RNAs were prepared from 70 day old adult mice testes (lanes 1, 4 and 7), spermatocytes (lanes 2, 5 and 8) and COP5 cells (lanes 3, 6 and 9). mRNAs (5 μg) were electrophoresed on 1.2% agarose−formaldehyde gels and analyzed by Northern hybridization with cDNAs of mouse lamin B_1 (**a**), mouse lamin B_2 (**b**), a 37 base mouse lamin C oligonucleotide of nucleotides 883−919 (**c**) and mouse DNA polymerase β (**d**). The filter hybridized with lamin C oligonucleotide was subsequently rehybridized with cDNA of DNA polymerase β to compare the amount of testis mRNA with spermatocyte mRNA (Hirose *et al.*, 1989). Panel (d) indicates that the two mRNAs were present in similar amounts. Lamin A (2.9 kb), B_1 (2.9 kb), B_2 (3.5 kb) and C (2.0 kb) genes are marked by A, B_1, B_2 and C, respectively. The DNA polymerase β gene (1.5 kb) is marked by polβ. The asterisk indicates a newly identified lamin B_2-related 1.8 kb mRNA.

shown in Figure 1, so we concluded that lamins B_1, B_2 and B-related proteins, but not lamins A and C, were expressed in spermatocytes.

cDNA cloning and characterization of 1.8 kb mRNA from spermatocytes

To clone the 1.8 kb mRNA from spermatocytes, we selected cDNA from cDNA libraries of spermatocytes using lamin

B_2 cDNA. Three independent clones were isolated from oligo(dT) or randomly primed spermatocyte cDNA libraries. Sequence analysis indicated that these cDNA clones were derived from the same mRNA. The largest showed a 1.7 kb cDNA insert (clone KF2; lodged with the EMBL, GenBank and DDBJ nucleotide sequence databases, under accession number D13455), suggesting that it was nearly full length. The open reading frame (ORF) of 1425 nucleotides encoded a polypeptide of 474 amino acids and molecular weight 53 265 daltons (Figure 3a). The 53 kDa protein resulting from the expression of cDNA clone KF2 in bacteria was also recognized by anti-fish lamin-specific antibody (Figure 4a) and was termed lamin B_3.

Nucleotide and amino acid sequences of lamin B_3 showed that its 3′-terminal 1.24 kb had the same sequence as lamin B_2, corresponding to the 3′-translated region. Variation in this region was thought to be due to polymorphism (Figure 3). Zewe *et al.* (1991) recently reported the nucleotide sequence of the mouse lamin B_2 gene and showed that this gene was present in the mouse genome as a single copy. By comparing the nucleotide sequence of lamin B_2 gene with the cDNA sequence of lamin B_3, it can be seen that the boundary between identity and variation of lamins B_2 and B_3 exists between exons 4 and 5 of the genomic sequence of lamin B_2 (Figure 3b), and that the cDNA sequence corresponding to exons 1−4 of lamin B_2 is replaced by a completely different sequence in lamin B_3 (Figure 3a and b). The ~1.5 kb 3′-untranslated region of the lamin B_2 transcript was found to be absent from lamin B_3 (Figure 3c). Based on these results, we conclude that

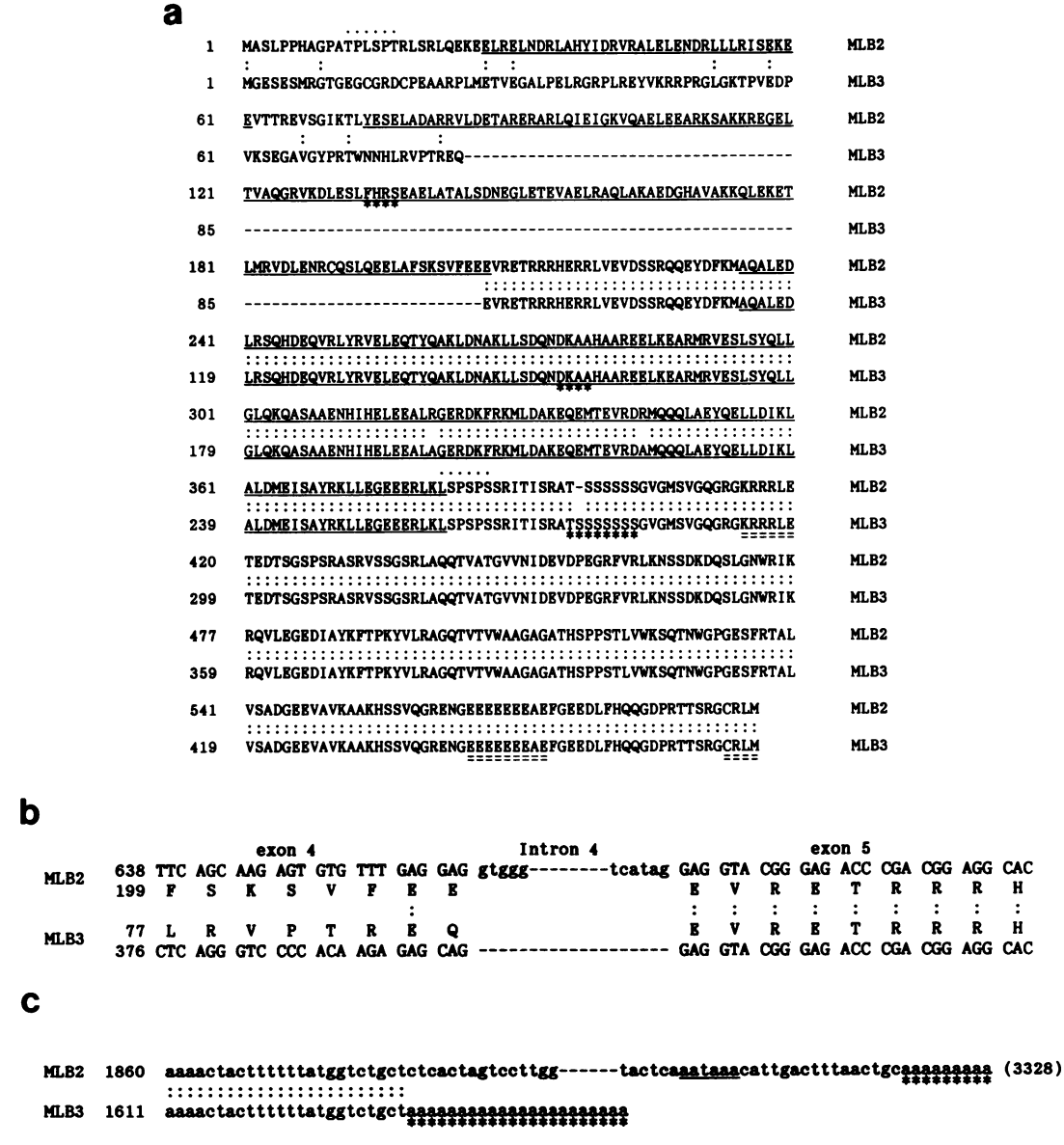

Fig. 3. Amino acid sequence comparison of murine lamins B_2 and B_3. (**a**) Lamin B_2 (MLB2) and deduced lamin B_3 (MLB3) protein sequences are indicated in the one-letter code. Identical amino acids are marked with double points. α-Helical coils 1A, 1B and 2 are underlined. The lamin conserved nuclear migration signal, acidic domain and C-terminal tetrapeptide motif, CxxM, are doubly underlined (Nigg, 1989). Three lamin B_2 specific sequence motifs are indicated by lines of asterisks (Höger *et al.*, 1990). Two sequence motifs that are phosphorylated by cdc2 kinase are indicated by dotted lines (Heald and McKeon, 1990). (**b**) and (**c**) The sequences of lamins B_2 and B_3 are shown for comparison around the intron 4−exon 4 and −exon 5 boundaries (shown in b) and of 3′ untranslated sequences (shown in c). The polyadenylation signal is underlined. Skipped sequences are indicated by broken lines. Intron nucleotide and untranslated sequences are indicated by small letters. Identical sequences are indicated by double points. Poly(A) tails are indicated by lines of asterisks.

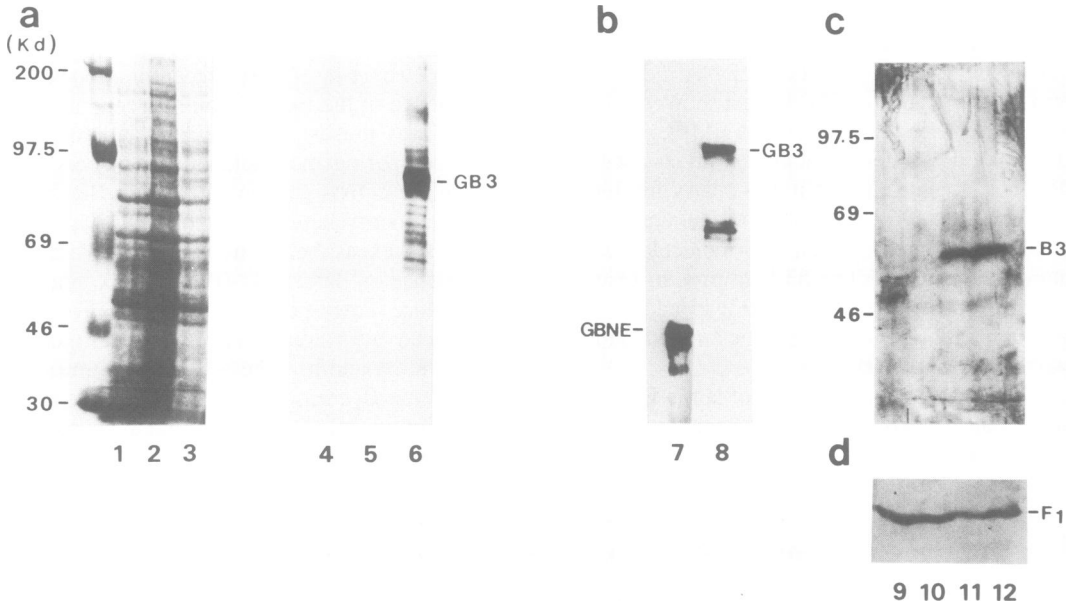

Fig. 4. Detection of lamin B_3 protein in spermatocytes by Western blot analysis. Total cell extracts (shown in **a**) and glutathione−Sepharose 4B affinity purified fractions (shown in **b**) were prepared from bacteria containing pGEX-2T (pG) (lanes 1 and 4), pG-B3NE (lanes 2, 5 and 7) and pG-B3 (lanes 3, 6 and 8). Total extracts (shown in **c** and **d**) were prepared from epididymal spermatozoa (lane 9), testes of 9 (lane 10) and 70 (lane 11) day old mice and purified pachytene spermatocytes (lane 12). The total cell extract proteins (30 μg, lanes 1−6; and 150 μg, lanes 9−12) and affinity purified proteins (50 ng) were electrophoresed on 10% SDS−polyacrylamide gels and analyzed by Western blotting with anti-fish lamin-specific antibody (lanes 4−6) and affinity purified anti-lamin B_3-specific antibody (lanes 7−12). Proteins were visualized by staining with Coomassie blue (lanes 1−3). Mouse tissue and cell extracts were also analyzed by Western blotting with anti-F_1-ATPase-specific antibody assessed as controls. Panel (**d**) indicates that the mitochondrial F_1-ATPase (F_1) is present in all lanes. The lower bands in lanes 7 and 8 are degradation products of the chimeric proteins. The chimeric lamin B_3 proteins are marked by GB3 and GBNE respectively, and lamin B_3 protein is marked by B3.

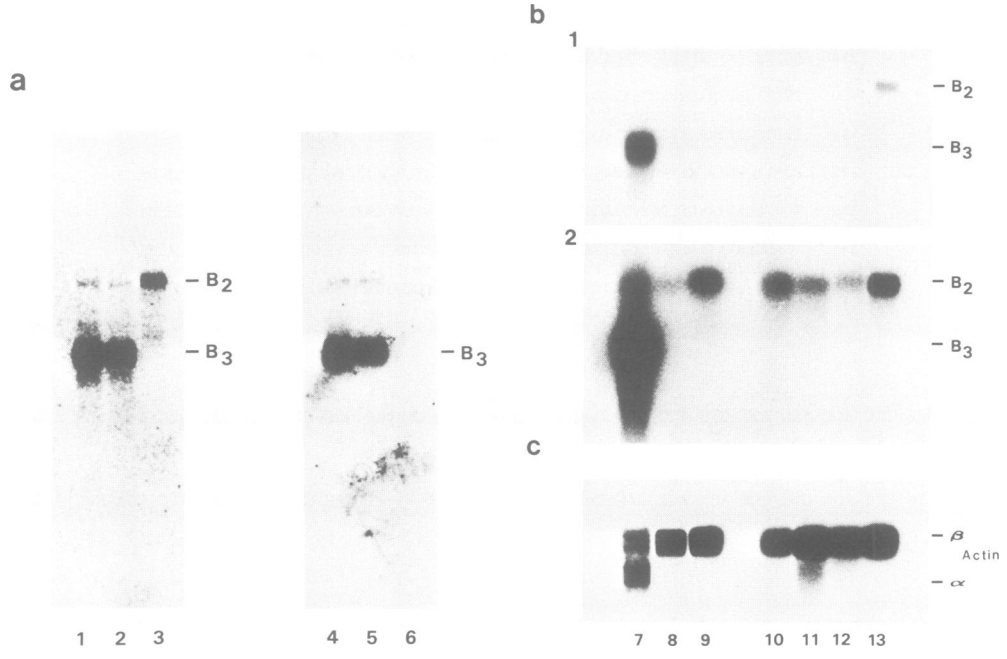

Fig. 5. Analysis of spermatocyte specific expression of lamin B_3 by Northern hybridization. (**a**) Poly(A)$^+$ RNAs were prepared from testes (lanes 1 and 4) and spermatocytes (lanes 2 and 5) of 70 day old adult mice and COP5 cells (lanes 3 and 6) and analyzed by Northern hybridization. The filter was first hybridized with whole cDNA of lamin B_3 (lanes 1−3), and subsequently rehybridized with the 5′-terminal lamin B_3 specific sequence of nt 1−379 (lanes 4−6). 5 μg of mRNA were electrophoresed on a 1.2% agarose−formaldehyde gel. (**b**) and (**c**) Poly(A)$^+$ RNAs were prepared from testes (lane 7), stomach (lane 8), kidney (lane 9), liver (lane 10), thymus (lane 11), spleen (lane 12) and brain (lane 13) of 70 day old adult mice, and analyzed by Northern hybridization. The filter was first hybridized with cDNA of lamin B_2 (b) and subsequently rehybridized with cDNA of mouse β actin (c). 10 μg of testis mRNA and 20 μg of other mRNAs were electrophoresed on a 1.2% agarose−formaldehyde gel. Panels b_1 and b_2 illustrate short and long exposures, respectively. Lamin B_2 and B_3 genes are marked by B_2 and B_3, respectively. Actin genes assessed as controls are marked by α and β Actin (Spiegelman *et al.*, 1983).

lamin B_3 is generated by lamin B_2 by differential splicing and alternative polyadenylation.

Comparison of the amino acid sequences of lamins B_2 and B_3

The amino acid sequence of lamin B_3 indicated that its C-terminal tail and coil 2 domains were identical to those of lamin B_2, these regions having a specific nuclear migration signal, an acidic domain and a C-terminal tetrapeptide motif, CxxM (Figure 3a; Nigg, 1989; McKeon, 1991). The 3 kDa N-terminal head domain and coil 1A and 1B domains of lamin B_2 were replaced by a 13 kDa amino acid sequence in lamin B_3 (Figures 3a and 6a). In nuclear lamin proteins, the conserved C-terminal TPLSPT residues of the head domain and the first 32 amino acid residues of the coil 1A domain (Figure 3a) are known to be involved in the disassembly and assembly respectively of the nuclear envelope (Heald and McKeon, 1990; Peter *et al.*, 1991). The 13 kDa domain of lamin B_3 did not have such conserved sequences and no homology in this domain was found with previously described proteins. However, nine proline residues appear semi-periodically in a segment corresponding to amino acids 19–80 of this domain (Figures 3a and 6a), so the expectation is that it is unable to form an α-helical structure. Lamin B_3 thus has a longer head domain and a shorter central rod domain than any of the other lamins.

Western blot analysis using an anti-lamin B_3-specific antibody

To confirm that a protein product derived from the mRNA of lamin B_3 exists in pachytene spermatocytes, anti-lamin B_3-specific polyclonal antibody was produced against the lamin B_3-specific N-terminal sequence of amino acids 1–76 (Figure 6a) fused to glutathione-S-transferase (Smith and Johnson, 1988). Total cell extracts from 9 and 70 day old mice testes, purified pachytene spermatocytes and epididymal spermatozoa were analyzed by Western blotting with affinity purified anti-lamin B_3-specific antibody. An ~55 kDa protein was recognized by this antibody in extracts from 70 day old mice testes and pachytene spermatocytes (Figure 4c). This value was roughly consistent with the 53 kDa protein predicted from the cDNA of lamin B_3 and the 51 kDa protein from Western blot analysis with anti-fish lamin-specific antibody (Figure 1b). In contrast, this protein was not detected in 9 day old mice testes and spermatozoa (Figure 4c). The 9 day old mouse testis is composed of Sertoli cells, spermatogonia and preleptotene spermatocytes (Bellvé *et al.*, 1977), so lamin B_3 protein is only present during meiosis. These results indicate that lamin B_3 is a nuclear matrix protein derived from pachytene-stage cells in the testis.

Northern blot analysis of various tissues

The expression of lamin B_3 was further investigated by Northern blot analysis using a lamin B_3 specific probe corresponding to the 5′-terminal sequence of lamin B_3 cDNA. We detected a 1.8 kb lamin B_3 mRNA in the mRNA from spermatocytes, but not in the mRNA from COP5 cells (Figure 5a, lanes 4–6). We also studied the expression of lamin B_3 in mouse tissues. While lamin B_2 mRNA was found in all the mouse tissues examined, lamin B_3 was detected only in testes (Figure 5b, panels 1 and 2). It was therefore concluded that lamin B_3 is spermatocyte specific.

Behavior of somatic cell nuclei transiently transfected with chimeric lamin B_3

To examine the intracellular distribution of lamin B_3 and to determine its effects on the cell cycle, we constructed a hybrid gene encoding a polypeptide (YPYDVPDYA) of hemagglutinin (HA) (Kolodziej and Young, 1989) joined to the N-terminal end of lamin B_2 and B_3 cDNAs (Figure 6a), and transfected it into COP5 cells which expressed the lamin B_2 gene (Figure 2, lane 6). Cells that transiently expressed the vector for epitope tagging (Figure 6b) were then detected by immunofluorescence using a monoclonal antibody (12C65) raised against the HA polypeptide (Niman *et al.*, 1983). The chimeric lamin B_3 was found to be concentrated in the nuclear envelope and nucleus during the

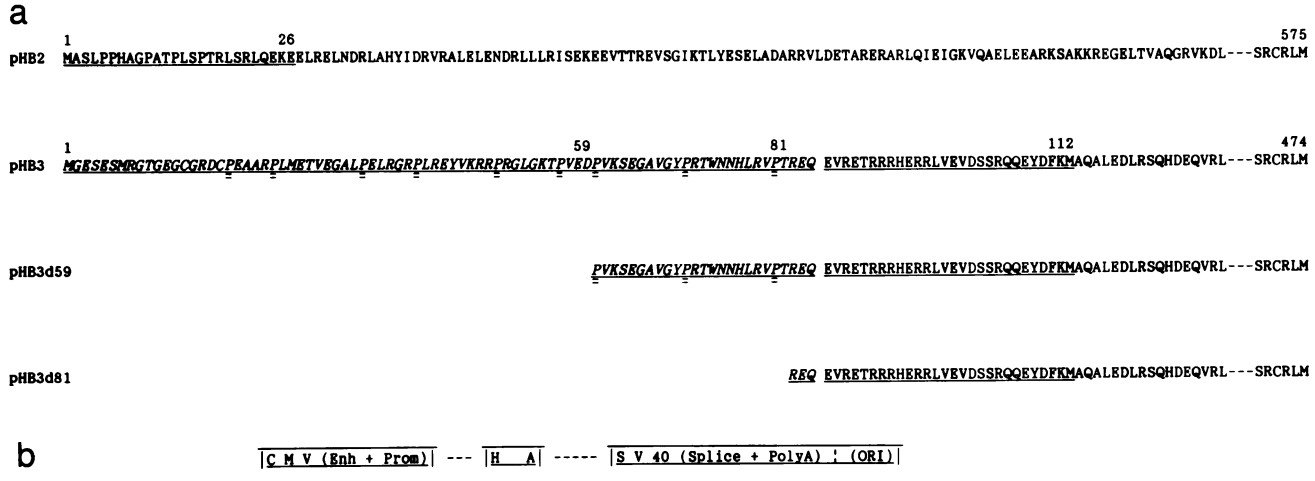

Fig. 6. Structure of chimeric lamin B_3 proteins and the expression vector for epitope tagging. The N-terminal amino acid sequences of lamin B_2 (pHB2) and B_3 (pHB3) and mutant lamin B_3 (pHB3d59 and pHB3d81) proteins are shown in (**a**). The structure of the derivative of expression vector pCDM8 (Seed, 1987) is shown in (**b**). The four purified fragments were inserted into 3′ multicloning sites of polypeptide YPYDVPDYA of HA (Kolodziej and Young, 1989). Transcription of chimeric DNAs is directed by the cytomegalovirus promoter–enhancer element [CMV (Enh + Prom)] element and is terminated by the SV40 splicing (Splice)/polyadenylation (Poly A) signals. The N-terminal head domains are underlined. Lamin B_3 specific N-terminal sequences are italicized. Nine proline residues are doubly underlined.

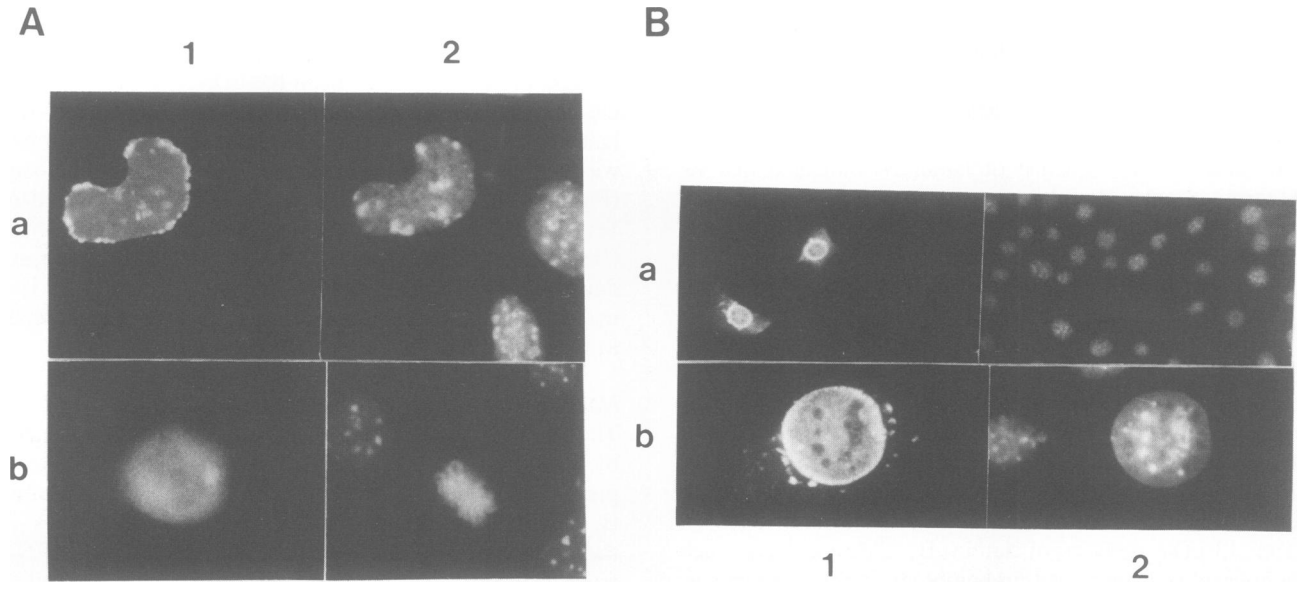

Fig. 7. Immunofluorescence localization of expressed chimeric lamins B_2 and B_3 in somatic COP5 cells. The cDNA of lamins B_2 and B_3 was tagged at the N-terminus with a sequence encoding influenza virus HA (Kolodziej and Young, 1989) and then transfected into COP5 cells. The product of pHB2 and B3 was detected using an anti-HA monoclonal antibody (Niman *et al.*, 1983). Panel (**A**): (a) and (b) show the distributions of chimeric lamin B_3 at interphase and metaphase respectively of the mitotic cell cycle. Panel (**B**) shows the distribution of chimeric lamin B_2 at interphase of the mitotic cell cycle. Staining with an FITC-labeled secondary antibody is shown in column 1; the same fields labeled with Hoechst dye 33258 to identify nuclei are shown in column 2.

interphase of the mitotic cell cycle (Figure 7A, panels a) and dispersed into the cytoplasm from the nucleus in metaphase (Figure 7B, panels b). This was similar to the distribution of the chimeric lamin B_2 assessed as controls (Figure 7B) and the behavior of endogenous somatic-type lamins (Gerace and Blobel, 1980; Stick *et al.*, 1988; Kitten and Nigg, 1991). The C-terminal domain of the lamin B_3, which is present in several specific amino acid motifs, as described above, can therefore function as in somatic-type lamins. In addition, we detected an interesting change in phenotype regarding the interphase nuclear morphology of COP5 cells made to express lamin B_3. Namely, the normal spherical shape became transformed to a hook shape by transfection of pHB3 (Figure 8a,b and e–h). This unusual phenotype has never been found in COP5 cells made to express the chimeric lamin B_2.

To determine which lamin B_3 specific N-terminal region was involved in this alteration of nuclear morphology, two chimeric lamin B_3 genes, pHB3d59 and pHB3d91, which were variously deleted within the N-terminal region (Figure 6a), were constructed. Their protein products, lacking amino acid residues 1–59 and 1–81 respectively, could also be translocated into nuclei in transfected cells, but did not induce morphological transformation of nuclei (Figure 8c,d and i–l). These results suggest that the lamin B_3 specific N-terminal sequence, and not deletion of the N-terminal sequence containing the head, coil1A and coil1B domains from lamin B_2, is responsible for the change in nuclear architecture.

Discussion

Lamin B_3 is a spermatocyte specific lamin

The results presented in this communication clearly demonstrate that a germ cell specific lamin exists in mouse spermatocytes. Hitherto, germ cell specific lamins L_{III} and L_{IV} had been detected only in *Xenopus* diplotene oocytes and spermatids, respectively (Stick, 1988; Benavente and Krohne, 1985). However, in both *Xenopus* and the chicken, lamins were reported to be absent in the pachytene stage gametocytes (Stick and Schwarz, 1982, 1983; Lehner *et al.*, 1987).

In mammals, previous work has not generated a clear picture concerning expression of lamins. Some workers have reported that nuclear lamins can be detected during gametogenesis (Schatten *et al.*, 1985; Maul *et al.*, 1986; Moss *et al.*, 1987; Sudhakar and Rao, 1990), while others have noted that the nuclear lamina is absent in spermatocytes at the ultrastructural level (Fawcett, 1966) and that nuclear lamins cannot be demonstrated by immunoblotting and immunostaining methods (Stick and Schwarz, 1982; Longo *et al.*, 1987; Kaufmann, 1989; Höger *et al.*, 1990). To clarify this problem we performed more direct studies using Northern blot analysis in addition to Western blot analysis. Both analyses clearly revealed that lamins B_1, B_2 and B_3 are present and can be regulated at the transcriptional level in spermatocytes (Figures 2 and 5). In contrast, lamins A and C were absent. Furthermore, Northern blotting and Western blotting of various tissues demonstrated that the expression of lamin B_3 is restricted to spermatocytes (Figures 2, 4 and 5), and thus the lamin B_3 first described in the present communication is a spermatocyte specific lamin.

Recently, Sudhakar *et al.* (1992), using biochemical and immunological methods, found a germ cell specific 60 kDa lamin B-related protein in rat pachytene spermatocytes, which could be detected in all stages of cells in spermatogenesis but not in the liver. While the sequence of this protein is not yet available, its molecular weight and immunological properties suggest that it is not identical to lamin B_3. Our results on lamin expression in spermatocytes are consistent with the findings of Moss *et al.* (1987), who showed that in addition to 67 kDa lamin B, a 55 kDa protein which reacts

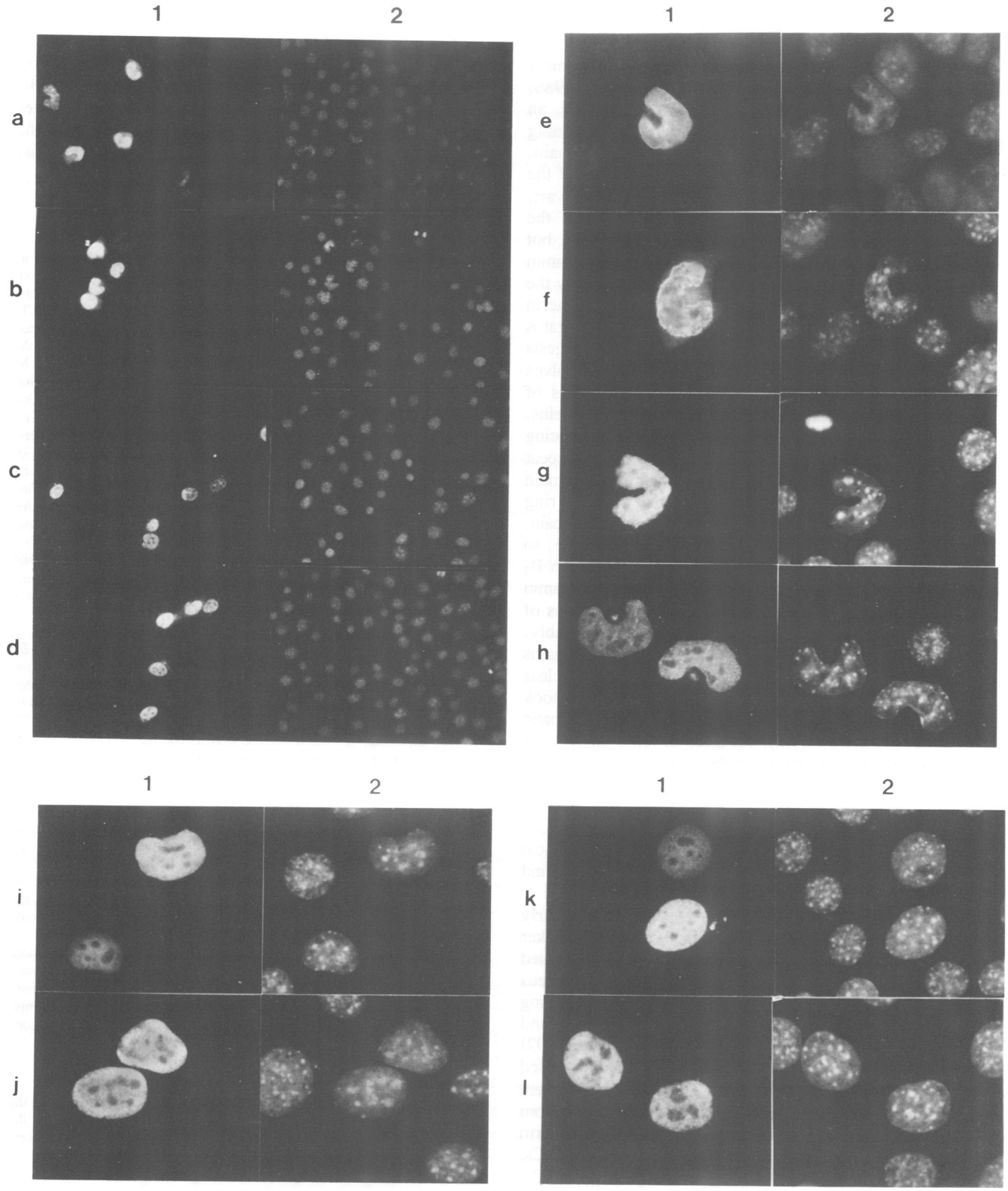

Fig. 8. Functional analysis by ectopic expression of serial N-terminal deleted lamin B_3 genes in somatic COP5 cells. COP5 cells were transfected with 20 μg DNA of serial N-terminal deleted constructs; pHB3 (**a**, **b** and **e**–**h**), pHB3d59 (**c**, **i** and **j**) and pHB3d81 (**d**, **k** and **l**). The fusion protein products were detected with an anti-HA monoclonal antibody. Staining with an FITC-labeled secondary antibody is shown in (1), whereas the same field labeled with Hoechst dye 33258 to identify nuclei is shown in (2).

with anti-rat lamin B-specific antibody is also present in pachytene spermatocytes. Although the sequence of this protein is also not available, it is possible that the 55 kDa protein and lamin B_3 may be identical on the bases of molecular weight, immunoreactivity against anti-lamin B-specific antibody and expression pattern. Thus, their results support our conclusion that lamin B_3 is present in the nuclear matrix from pachytene spermatocytes.

Lamin B_3 is involved in assembly of specific nuclear architectural features

Lamin proteins are members of the intermediate filament (IF) protein family (Aebi *et al.*, 1986; Fisher *et al.*, 1986; McKeon *et al.*, 1986), whose structures comprise an N-terminal head domain, a central rod domain containing α-helical coils 1A, 1B and 2, and a C-terminal tail domain. Each of these domains is important for the function of the lamins (Steinert and Roop, 1988; Quinlan and Stewart, 1991). Georgatos *et al.* (1988) have reported that the common N-terminal head domains of lamins A and C, but not the C-terminal tail domains, interacted directly with lamin B in an *in vitro* binding assay. On the other hand, only the C-terminal tail domains of desmin, vimentin and peripherin bind to lamin B (Georgatos and Blobel, 1987; Georgatos *et al.*, 1987; Djabali *et al.*, 1991). This evidence suggests that the integrity of nuclear envelope assembly involves polarity associations between hetero- and homo-types of A-type and B-type lamins and other cytoplasmic IF proteins.

On the basis of the result from the amino acid sequencing of lamin B_3, we know that there is a semi-periodical repeat of nine proline residues in the N-terminal head domain (Figures 3a and 6a). A proline residue that forms a ring structure produces a significant bend in the amino acid chain. Lamin B_2 does not have such an amino acid sequence, so it can be concluded that the conformation of the lamin B_3 N-terminal head domain is highly different from that of lamin B_2; these differing polarities could allow the two types of lamin to exhibit distinct properties in filament assembly. Indeed a functional difference in the N-terminal domains of lamin B_2 and B_3 is evidenced by effects on nuclear morphology with a transformation from spherical to hook shape only being found in lamin B_3-expressing somatic COP5 cells (Figure 8e–h). Further, deletion analysis of lamin B_3 clearly demonstrated that the N-terminal head domain of lamin B_3 is responsible for this alteration (Figure 8i–l). This evidence strongly suggests that the nuclear architecture is different between somatic and meiotic cells at the molecular level, in line with previous histological observations (Counce and Meyer, 1973; Fawcett and Chemes, 1979; Heyting *et al.*, 1985).

The molecular function of lamin B_3 in meiosis clearly requires further investigation. In meiosis, however, Sudhakar and Rao (1990) have described that a 60 kDa lamin B-related protein is redistributed from the periphery of the nucleus into synaptonemal complexes (SCs) at prophase during meiosis in the rat. In addition, Heyting *et al.* (1987) found 30 and 33 kDa proteins and Smith and Benavente (1992) found a 48 kDa protein using monoclonal antibodies raised against SCs from rat spermatocytes. They were only present in SC-residual structures of the nuclear matrix fraction from pachytene spermatocytes. These findings suggest that germ cell specific nuclear matrix proteins are a component of SCs. Therefore lamin B_3 might also be a component of SCs. Moreover, morphological examinations have revealed that SCs are present in pachytene chromosomes at the axes associated with the nuclear membrane at their ends (Woollam *et al.*, 1966; Esponda and Gimènez-Martin, 1972; Heyting *et al.*, 1985). When gametocytes enter into meiotic prophase, the pairing of homologous chromosomes is initiated near the attachment sites of SCs (Counce and Meyer,1973), which might therefore be directly involved in the assembly of pachytene chromosomes. Lamins A and C have already been demonstrated to bind directly to mitotic chromosomal surfaces and assemble the nuclear envelope with lamin B (Burke and Gerace, 1986; Burke, 1990; Glass and Gerace, 1990; Yuan *et al.*, 1991). It is thus possible that lamin B_3, which induces a hook shape in somatic nuclei, participates in reorganization of pachytene chromosomes rather than the nuclear matrix at meiotic prophase during spermatogenesis.

Materials and methods

Isolation of nuclear matrix proteins

Pachytene spermatocytes were prepared from 70 day old adult mice as described previously (Chandley *et al.*, 1977). Nuclear matrix fractions from whole testes and spermatocytes were prepared by the following treatments. All nuclear isolation solutions contained final concentrations of 1 mM DTT, 1 mM PMSF, 0.15 mM spermine and 0.5 mM spermidine. Decapsulated testes were finely fragmented using forceps in buffer I (10 mM HEPES–NaOH at pH 7.6, 25 mM KCl, 1 mM EDTA and 0.25 M sucrose), and then dispersed in a blender at low speed. After centrifugation for 10 min at 800 *g*, the testicular cells and spermatocytes were suspended in buffer A (10 mM HEPES–NaOH at pH 7.6, 1.5 mM $MgCl_2$ and 10 mM KCl), lysed in a Dounce homogenizer and re-centrifuged to pellets. The crude nuclei, well suspended in buffer I, were underlaid with buffer II (10 mM HEPES–NaOH at pH 7.6, 25 mM KCl, 11 mM EDTA and 2.0 M sucrose) and centrifuged for 60 min at 51 000 *g* to obtain purified nuclei. These were digested with DNase I and RNase A in buffer A for 30 min at 22°C. After centrifugation for 10 min at 800 *g*, digested nuclear pellets were resuspended in buffer I containing 2% Triton X-100 and then centrifuged. The final nuclear matrix fractions were prepared by washing with 2 M NaCl in buffer I. Total cell extracts and nuclear matrix proteins were prepared by sonication in buffer E (50 mM Tris–HCl at pH 8.8 containing 0.4 M NaCl, 0.5% SDS and 8 M urea).

Northern hybridization and screening of cDNA encoding lamin B_3

The total RNAs of COP5 cells (Tyndall *et al.*, 1981), spermatocytes and testes were extracted with guanidium thiocyanate solution and precipitated by centrifugation in cesium chloride solution (Sambrook *et al.*, 1989). Poly(A)$^+$ RNAs were purified from total RNAs using Oligotex-dT30 (Roche Ltd). Electrophoresed mRNAs were transferred to nylon membranes by the capillary method and immobilized by UV irradiation. Hybridizations were performed at 42°C in 6 × SSC (20 × SSC: 3 M NaCl, 0.3 M sodium citrate at pH 7.0) containing 50% (v/v) deionized formamide, 1% SDS, 5 × Denhardt's reagent and 50 μg/ml salmon sperm DNA with ^{32}P-radiolabeled probes. The cDNAs were labeled by random priming. The 37 base oligonucleotide was labeled by phosphorylation using T4 polynucleotide kinase. Nylon membranes were hybridized with cDNA or oligonucleotide washed in 0.1 × SSC containing 0.1% SDS at 65°C or at the melting temperature. The hybridized probes were removed in 10 mM sodium phosphate at pH 6.5 containing 50% (v/v) deionized formamide at 65 or 75°C.

A λ-ZAP II cDNA library was constructed using oligo(dT) and random primers on poly(A)$^+$ RNA from purified mouse pachytene spermatocytes. In order to obtain a clone coding for lamin B_3, 5 × 10^6 recombinant plaques were screened with whole lamin B_2 cDNA fragments. Hybridization and washing were performed under the same conditions described above.

DNA sequencing

Unidirectional deletion derivatives of KF clones were constructed, and the nucleotide sequence of each clone was determined in both orientations by the dideoxy sequencing method using a Pharmacia LKB ALF DNA sequencer.

Construction of lamin B_2 and lamin B_3 expression plasmids

The parental plasmid pZAP-LB_2 was used for generating a lamin B_2 ORF fragment (B2), corresponding to nucleotides (nt) 42–1840 (*Nco*I fragment). The pZAP-KF2 was used for generating a set of fragments of lamin B_3. To generate the complete ORF fragment (B3, corresponding to nt 150–1597), the lamin B_3 specific N-terminal fragment (B3NE, corresponding to nt 150–379) and the first N-terminal deleted lamin B_3 fragment (B3d59, corresponding to nt 325–1597), pZAP-KF2 was digested at the *Nco*I sites, the *Nco*I+*Eco*81I sites and the *Bam*HI+*Nco*I sites respectively. To generate the second N-terminal deleted lamin B_3 (B3d81, corresponding to nt 393–1597), pZAP-KF2 was digested at the *Xba*I and *Bst*XI sites located in the 5′ multicloning sites of lamin B_3 ORF, followed

by treatment with the exonuclease III and mung bean nuclease method (see the instruction manual of λ-ZAP II, Stratagene).

To construct eukaryotic expression vectors, the four purified fragments (B2, B3, B3d59 and B3d81) were inserted into 3′ multicloning sites of a derivative of expression vector pCDM8 (Seed, 1987) containing a gene encoding nine amino acids of the influenza virus HA (Kolodziej and Young, 1989).

To construct prokaryotic expression vectors, the two purified fragments (B3 and B3NE) were inserted into the 3′ multicloning sites of pGEX-2T (pG) containing a gene encoding glutathione-S-transferase (Smith and Johnson, 1988).

Antibody

The lamin B_3 specific N-terminal sequence, fused to the C-terminus of 26 kDa glutathione-S-transferase, was prepared by glutathione−Sepharose 4B affinity chromatography and SDS−PAGE from a crude extract of bacteria transformed with pG-B3NE (Smith and Johnson, 1988). Polyacrylamide gel containing this chimeric protein was directly injected into rabbits to produce the anti-lamin B_3-specific polyclonal antibody. Affinity purified anti-lamin B_3-specific antibody was prepared by the immunoblot method from rabbit serum that had been adsorbed to remove non-specific and glutathione-S-transferase binding proteins with acetone powders from mouse liver and bacteria transformed with pGEX-2T. The details of the immunological methods applied are described by Harlow and Lane (1988).

An anti-fish lamin-specific monoclonal antibody raised against lamins from rainbow trout erythrocytes and an anti-F_1-ATPase-specific polyclonal antibody raised against chloroplast F_1-ATPase (Mukohata *et al.*, 1987) from *Spinacia* were generously provided by Drs Yamaguchi and Nagahama of the National Institute for Basic Biology and Dr Mukohata of the Department of Biology in Nagoya University, respectively.

Immunofluorescence

Localization of the chimeric lamin proteins was detected with FITC using the following method. COP5 cells were fixed in phosphate-buffered saline (PBS) containing 3.5% formaldehyde, and made permeable with 0.1% NP-40 at room temperature (Satake and Luftig, 1983). The cells were incubated with monoclonal antibody 12C56 (Niman *et al.*, 1983) raised against the nonapeptide (YPYDVPDTA) of HA [1:500 in PBS with 1% bovine serum albumin (BSA) for 1 h at 37°C], and then incubated with FITC-labeled rabbit anti-mouse immunoglobulin (1:50 in PBS with 1% BSA for 1 h at 37°C). DNA was stained with Hoechst 33258.

DNA transfection

Plasmid DNAs were transfected into COP5 cells in 10 cm dishes with coverglasses by the calcium phosphate coprecipitation method as described previously (Furukawa *et al.*, 1990).

Polyacrylamide gel electrophoresis and Western blotting

Protein samples were run on SDS−polyacrylamide gels (Laemmli, 1970) and analyzed by Western blotting as described by Towbin *et al.* (1979).

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References

Aebi,U., Cohn,J., Buhle,L. and Gerace,L. (1986) *Nature*, **323**, 560−564.
Bellvé,A.R., Cavicchia,J.C., Millette,C.F., O'Brien,D.A., Bhatnagar,Y.M. and Dym,M. (1977) *J. Cell Biol.*, **74**, 68−85.
Benavente,R. and Krohne,G. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6176−6180.
Burke,B. (1990) *Exp. Cell Res.*, **186**, 169−176.
Burke,B. and Gerace,L. (1986) *Cell*, **44**, 639−652.
Chandley,A.C., Hotta,Y. and Stern,H. (1977) *Chromosoma*, **62**, 243−253.
Counce,J.S. and Meyer,G.F. (1973) *Chromosoma*, **44**, 231−253.
Djabali,K., Portier,M.-M., Gros,F., Blobel,G. and Georgatos,S.D. (1991) *Cell*, **64**, 109−121.
Esponda,P. and Gimènez-Martin,G. (1972) *Chromosoma*, **38**, 405−417.
Fawcett,D.W. and Chemes,H.E. (1979) *Tissue and Cell*, **11**, 147−162.
Fisher,D.Z., Chaudhary,N. and Blobel,G. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 6450−6454.
Furukawa,K., Yamaguchi,Y., Ogawa,E., Shigesada,K., Satake,M. and Ito,Y. (1990) *Cell Growth Differ.*, **2**, 135−147.
Gerace,L. and Blobel,G. (1980) *Cell*, **19**, 277−287.
Gerace,L. and Burke,B. (1988) *Annu. Rev. Cell Biol.*, **4**, 335−374.
Georgatos,S.D. and Blobel,G. (1987) *J. Cell Biol.*, **105**, 117−125.
Georgatos,S.D., Weber,K., Geisler,N. and Blobel,G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6780−6784.
Georgatos,S.D., Stournaras,C. and Blobel,G. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4325−4329.
Glass,J.R. and Gerace,L. (1990) *J. Cell Biol.*, **111**, 1047−1057.
Harlow,E. and Lane,D. (1988) *Antibodies. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Heald,R. and McKeon,F. (1990) *Cell*, **61**, 579−589.
Heyting,C., Dietrich,A.J.J., Redeker,E.J.W. and Vink,A.C.G. (1986) *Eur. J. Cell Biol.*, **36**, 307−314.
Heyting,C., Moens,P.B., Raamsdonk,W.v., Dietrich,A.J.J., Vink,A.C.G. and Redeker,E.J.W. (1987) *Eur. J. Cell Biol.*, **43**, 148−154.
Hirose,F., Hotta,Y., Yamaguchi,M. and Matsukage,A. (1989) *Exp. Cell Res.*, **181**, 169−180.
Höger,T.H., Krohne,G. and Franke,W.W. (1988) *Eur. J. Cell Biol.*, **47**, 283−290.
Höger,T.H., Zatloukal,K., Waizenegger,I. and Krohne,G. (1990) *Chromosoma*, **99**, 379−390.
Holz,D., Tanaka,R.A., Hartwig,J. and McKeon,F. (1989) *Cell*, **59**, 969−977.
Kaufmann,S.H. (1989) *J. Biol. Chem.*, **264**, 13946−13955.
Kitten,G.T. and Nigg,E.A. (1991) *J. Cell Biol.*, **113**, 13−23.
Kolodziej,P. and Young,A. (1989) *Mol. Cell. Biol.*, **9**, 5387−5394.
Krohne,G. and Benavente,R. (1986) *Exp. Cell Res.*, **162**, 1−10.
Krohne,G., Waizenegger,I. and Höger,H.T. (1989) *J. Cell Biol.*, **109**, 2003−2011.
Laemmli,U.K. (1970) *Nature*, **227**, 680−685.
Lebel,S., Lamporn,C., Royal,A. and Raymond,Y. (1987) *J. Cell Biol.*, **105**, 1099−1104.
Lehner,C.F., Stick,R., Eppenberger,H.M. and Nigg,E.A. (1987) *J. Cell Biol.*, **105**, 577−587.
Loewinger,L. and McKeon,F. (1988) *EMBO J.*, **7**, 2301−2309.
Longo,F.J., Krohne,G. and Franke,W.W. (1987) *J. Cell Biol.*, **105**, 1105−1120.
Maul,G.G., French,B.T. and Bechtol,K.B. (1986) *Dev. Biol.*, **115**, 68−77.
McKeon,F. (1991) *Curr. Opin. Cell Biol.*, **3**, 82−86.
McKeon,F., Kirschner,M.W. and Caput,D. (1986) *Nature*, **319**, 463−468.
Moss,S.B., Donovan,M.J. and Bellve,A.R. (1987) *Ann. N.Y. Acad. Sci.*, **513**, 74−89.
Mukohata,Y., Ihara,K., Yoshida,M., Konishi,J., Sugiyama,Y. and Yoshida,M. (1987) *Arch. Biochem. Biophys.*, **256**, 650−653.
Niman,H.L., Houghten,R.A., Walker,L.E., Reisfeld,R.A., Wilson,I.A., Hogle,J.M. and Lerner,R.A. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4949−4953.
Nigg,E.A. (1989) *Curr. Opin. Cell Biol.*, **1**, 435−440.
Peter,M., Heitlinger,E., Hàner,M., Aebi,U. and Nigg,E.A. (1991) *EMBO J.*, **10**, 1535−1544.
Quinlan,R.A. and Stewart,M. (1991) *BioEssays*, **13**, 597−600.
Riedel,W. and Werner,D. (1989) *Biochim. Biophys. Acta*, **1008**, 119−122.
Röber,R.-A., Weber,K. and Osborn,M. (1989) *Development*, **105**, 365−378.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Satake,M. and Luftig,R. (1983) *Virology*, **124**, 259−273.
Schatten,G., Maul,G.G., Schatten,H., Chaly,N., Simerly,C., Balczon,R. and Brown,D.L. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4727−4731.
Seed,B. (1987) *Nature*, **329**, 840−842.
Smith,A. and Benavente,R. (1992) *Exp. Cell Res.*, **198**, 291−297.
Smith,D.B. and Johnson,K.S. (1988) *Gene*, **67**, 31−40.
Spiegelman,B.M., Frank,M. and Green,H. (1983) *J. Biol. Chem.*, **258**, 10083−10089.
Steinert,P.M. and Roop,D.R. (1988) *Annu. Rev. Biochem.*, **57**, 593−625.
Stewart,C. and Burke,B. (1987) *Cell*, **51**, 383−392.

Stick,R. (1988) *EMBO J.*, **7**, 3189–3197.
Stick,R. and Schwarz,H. (1982) *Cell Differ.*, **11**, 235–243.
Stick,R. and Schwarz,H. (1983) *Cell*, **33**, 949–958.
Stick,R., Angres,B., Lehner,C.F. and Nigg,E.A. (1988) *J. Cell Biol.*, **107**, 397–406.
Sudhakar,L. and Rao,M.R.S. (1990) *J. Biol. Chem.*, **265**, 22526–22532.
Sudhakar,L., Sivakumar,N., Behal,A. and Rao,M.R.S. (1992) *Exp. Cell Res.*, **198**, 78–84.
Towbin,H., Staehelin,M. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Tyndall,C., La Mantia,G., Thacker,C.M., Favaloro,J. and Kamen,R. (1981) *Nucleic Acids Res.*, **9**, 6231–6250.
Woollam,D.H.M., Ford,E.H.R. and Millen,J.W. (1966) *Exp. Cell Res.*, **42**, 657–661.
Yuan,J., Simos,G., Blobel,G. and Georgatos,S.D. (1991) *J. Biol. Chem.*, **266**, 9211–9215.
Zewe,M., Höger,H.T., Fink,T., Lichter,P., Krohne,G. and Franke,W.W. (1991) *Eur. J. Cell Biol.*, **56**, 342–350.

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